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Ornithine Decarboxylase in Rat Skin

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Ornithine decarboxylase (ODC; E.C.4.1.1.17) activities can be stimulated 2–10 fold in rat epidermis and dermis by hair plucking. Stimulation does not involve the removal of a soluble ODC inhibitor. ODC activity in the dermis and whole skin decreased with aging, while the epidermis showed little change. The apparent Km for ornithine and the heat stability of ODC in plucked and unplucked skin were similar.

ODC was assayed in plucked and unplucked skin of rats fed diets containing between 2 and 24% protein. Activities in both plucked and unplucked skin were higher in the animals fed diets with higher protein contents. ODC levels were positively correlated with the weight changes undergone by rats on controlled-protein diets. In animals restricted to 2% protein diets and rehabilitated with 16% protein diets, enzyme levels were increased after 2 days rehabilitation and peaked after 5 days rehabilitation. The responsiveness of ODC to changes in dietary protein may be useful in the diagnosis of protein malnutrition.

Ornithine decarboxylase (E.C.4.1.1.17) (ODC) is the rate-limiting enzyme for the synthesis of the polyamines spermidine and spermine [1]. Increases in ODC activity are correlated with growth, specifically with tumorigenesis [2–4], liver regeneration [5], stimulation of lymphocytes by mitogens [6], stimulation of cells by epidermal growth factor [7] or growth hormone [8,9], and feeding of diets high in amino acids [10]. Decreases in ODC activities occur with administration of actinomycin D [11], cycloheximide [12] and during senescence [13]. The functions of polyamines in vivo are not known, although *in vitro* they can stabilize ribosomes [14] promote binding of mRNA [14] and increase RNA synthesis [12].

The skin is an ideal organ in which to study the factors regulating ODC. ODC activities can be correlated with the continuous growth of the dermis and epidermis. This type of growth can be altered by protein restriction, concurrently affecting ODC. In these studies, we manipulated growth of the whole skin by feeding rats on diets containing between 2 and 24% protein. Growth of hair follicles is more complex. Growth during the anagen phase of the hair cycle is analogous to the continuous growth of the whole skin. The transition of a follicle from the telogen (resting) state to anagen may be analogous to other processes in which ODC has been linked to the initiation of growth. We initiated follicular proliferation by plucking hair from telogen or catagen follicles.

In this paper we describe the chemical properties of rat skin ornithine decarboxylase and the effects of diet, aging and hair plucking on epidermis, dermis or whole skin ODC.

MATERIALS AND METHODS

DL-[1-^{14}C]-ornithine, uniformly labeled L-[^{14}C (U)]-ornithine, [1,4-^{14}C]-putrescine, phenethylamine and Aquasol were obtained from New England Nuclear (Boston, Ma.). Pyridoxal phosphate, Trizma base, bovine serum albumin and L-ornithine were from Sigma (St. Louis, Mo.). Protein-free diet mix, casein and vitamin supplement were purchased from ICN Pharmaceuticals (Cleveland, Oh.). EDTA and powdered human hemoglobin were from Matheson, Coleman and Bell (Norwood, Oh.). Dithiothreitol was from Bachem Feinchenikalien (Liestal, Switzerland). Carboxymethyl cellulose paper was from Whatman.

Animals

Male rats (CD strain) were purchased from Charles River and used, unless otherwise noted, when weighing 100–150 gm. Pre-weanling rats were not sexed. Rats were maintained, 1–3 per cage, on a 12 hr light-12 hr dark cycle and were fed ad libitum on diets of Purina Laboratory Chow (minimum 23% protein) and water prior to being placed on any specified diet.

Diet Experiments

Rats on controlled protein were fed *ad libitum* on mixtures of casein, protein-free diet and vitamins. In each diet the percentage by weight of vitamins was the same (2%); the percentage of protein-free diet was varied to balance differences in casein (e.g. a 12% diet contained 12% casein, 2% vitamin mix and 86% protein-free diet mix). The protein-free diet mix was composed of: 70% corn starch, 15% alphacel, 10% vegetable oil, 4% salt mixture U.S.P. XIV and 1% cod liver oil. All diets were isocaloric. We recorded the change in each rat's weight during its term on a special diet. In preliminary experiments animals were maintained on these diets for 3 days; more detailed studies were performed for 4 days or 8 days.

Standard Assay of ODC

Rats were anesthetized with ether, weighed, and all hairs were plucked from the dorsal posterior skin (ca. 4 × 4 cm) with a hemostat. The rats were returned to their cages, with food and water, and were killed by decapitation 4 hr later. We chose this time interval because with other methods of ODC stimulation, the enzyme peaks 4 hr after application of the stimulus [1,5]. The plucked skin and a more anterior patch of unplucked skin, which was plucked post mortem, were excised. The skin samples were cleaned of underlying fat and muscle, weighed, then minced and homogenized in 9 volumes of cold buffer (10 mM Tris, 5 mM DTT, 0.1 mM pyridoxal phosphate, 0.5 mM ethylene diaminetetraacetic acid [EDTA], pH 7.0) for 1.5 min with a polytron homogenizer (Brinkmann Instruments) in an ice slurry. Homogenates were centrifuged for 45 min at 27,000 × g in an RC-5 centrifuge and supernatants were used as the enzyme sources. Enzyme assays were run in 13 × 150 mm pyrex tubes containing 1 ml homogenate, 0.1 ml buffer (above) and 0.2 ml ornithine substrate (0.035 umoles DL-[1-^{14}C]-ornithine and 1.29 mmoles nonradioactive L-ornithine). Reactions were begun by addition of substrate; tubes were stoppered and incubated at 37° for 1 hr in a shaking water bath. Reactions were stopped by injecting 1 ml of 2 M citric acid. $^{14}CO_2$ was trapped in plastic wells containing 0.4 ml of phenethylamine that were suspended above the reaction mixtures. After equilibration for ½ hr, wells were removed, placed in scintillation vials with 5 ml of Aquasol and counted for radioactivity in a Packard 3375 spectrometer. Tubes containing only buffer and substrate were

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Abbreviations:
EDTA: ethylene diamine tetraacetic acid
DTT: dithiothreitol
ODC: ornithine decarboxylase

also incubated and their activities subtracted from each experimental measurement. All assays were done in duplicate and results are expressed as nanomoles CO_2 released/gm wet weight/hr, abbreviated "nanomles/gm/hr" in the tables and figures.

Localization of ODC

Six 3-day old rats were used for ODC localization in neonatal skin. Each skin was placed in buffer at 56° for 30 sec, then plunged immediately into ice and the epidermis was peeled from the dermis. The 6 specimens of dermis were pooled and the 6 specimens of epidermis were pooled and each pool was homogenized and assayed by the standard procedure.

A similar experiment was done with adult rats to determine the tissues that respond to plucking. A dorsal patch of skin was plucked; 4 hr later the rat was killed and plucked and unplucked skin removed. In each sample, dermis and epidermis were separated by heating. The skin layers were homogenized and ODC was assayed in each layer by the standard method. The experiment was done 4 times: once with a single rat and 3 times using pools of epidermis and dermis from 2 or 3 rats.

ODC was assayed in plucked skin and in several patches of unplucked skin, at various distances from the plucked area, to determine if plucking affects only plucked areas of skin.

Heat Stability

We tested the stability at 56° of ODC from plucked and unplucked skin. Homogenates of plucked and unplucked skin were incubated at 56°, and samples were withdrawn after 0, 5, 10, 15, 20 and 30 min. Stability was expressed as the percent of activity relative to the activity in the sample incubated for 0 min. The 2 homogenates were from different rats, each of which was fed Purina Chow.

Mixing Experiment

We tested for a soluble ODC inhibitor in unplucked skin. Homogenates of plucked and unplucked skin from a rat fed on a 16% casein diet were assayed by standard methods (1 ml homogenate included in each assay mix). One reaction vessel was prepared with 0.5 ml of each homogenate. The activity in the latter vessel was compared to the average of the activities in the first 2 vessels; less than the average activity would be taken as evidence of a diffusible ODC inhibitor in unplucked skin.

Stoichiometry of Reaction Products

To determine the ratio of CO_2 to putrescine formed from labeled ornithine, the procedure of Clark [15] was used with minor modifications. The principle of that method is that both ornithine and putrescine will stick to carboxymethyl-cellulose paper but only ornithine will be eluted by an "elution buffer" (10 mM Tris-HCl, 3 mM NaCl, pH 8.8–8.9).

We incubated 4 reaction mixtures: (1) an experimental mixture containing 1.28 μmoles unlabeled L-ornithine, 0.4 μCi uniformly labeled L-ornithine and 1 ml homogenate of plucked skin, (2) a mixture for measuring putrescine yields, containing 1.28 μmoles unlabeled L-ornithine, 0.313 μCi [1,4-^{14}C]-putrescine and 1 ml homogenate, (3) a mixture for measuring CO_2 production, containing 1.28 μmoles unlabeled L-ornithine, 0.4 μCi uniformly labeled L-ornithine and 1 ml homogenate, (4) a mixture for measuring ornithine yields, containing 1.6 μCi DL-[1-^{14}C]-ornithine, 1.28 μmoles unlabeled L-ornithine and 1 ml homogenate. Each mixture was spotted on CM-cellulose paper. The papers were dried, washed 10 times with 50 ml elution buffer (ca. 10 min per wash), dried again, and radioactivity was eluted by two 15-min washes with 50 ml of 1 N HCl. The HCl washes were rotary evaporated to small volumes, and aliquots were removed and counted for radioactivity. High voltage electrophoresis (pH 3.7, formic acid-pyridine, 2000 volts, 20 min) of these aliquots and putrescine, spermine, spermidine and ornithine standards was performed on the remainders of the HCl concentrates to determine the fractions containing radioactivity.

Histology

Samples of skin from rats subjected to various treatments were fixed in formalin. Sections 10 μ thick were cut, stained in hematoxylin and eosin, and examined under the light microscope.

Statistics

Statistics were performed using a 2-tailed student *t*-test, and results are expressed as means ±1 SD.

Protein Assays

Soluble proteins were assayed by 2 methods: (1) that of Schaffner and Weissman [16], where binding of Amidoschwartz to trichloroacetic acid-precipitated protein is measured; and (2) with Bio Rad protein dye, which quantitates the absorbance at 595 nm of a Coomassie blue-protein complex based on the technique of Bradford [17]. Bovine serum albumin was used as the standard. Hemoglobin was estimated by measuring absorbances at 540 nm, with powdered human hemoglobin in the reaction buffer as a standard.

RESULTS

Protein Assays

The method of Schaffner and Weissman [16] yielded protein contents of 10.4 ± 3.2 mg/gm skin (N = 24) in unplucked skin and 15.0 ± 4.6 (N = 24) in plucked skin. Homogenates assayed by the Coomassie blue method [17] had 24 ± 7.1 mg/gm protein (N = 18) in unplucked and 34.0 ± 5.9 mg/gm protein (N = 18) in plucked skin. For each method, the average protein content of plucked skin is statistically significantly higher ($p < 0.001$) than the protein content of unplucked skin. The ratio of the protein content of unplucked skin to the protein content of plucked skin from the same animal was 7.3 ± 2.0 for the 24 animals assayed according to Schaffner and Weissman [16] and 7.4 ± 2.4 for the 18 animals assayed with the Coomassie blue method [17]. Thus the ratio of unplucked to plucked protein content was independent of the methods used. Rarely (N = 1 in each group) was there more protein in an unplucked skin homogenate than in a plucked one.

Hemoglobin determinations for a series of unplucked-plucked pairs of homogenates indicated 7.1 ± 2.1 (N = 10) mg/gm for unplucked and 11.0 ± 3.0 (N = 10) mg/gm for plucked samples. In virtually every case, plucked skin contained more hemoglobin. In the rare cases where A_{540} was greater for the unplucked sample, the plucked samples had a distinctly more reddish color, and cloudiness of the unplucked samples appears to account for their greater absorbance. The difference between plucked and unplucked skin was statistically significant ($p < 0.001$). Because the hemoglobin contents of the homogenates were sufficiently high and variable to adversely affect protein assays, we have, except where noted, expressed our enzyme activities per gram (wet weight) of tissue.

Plucking

ODC activity in plucked skin was generally 2–10 times as high as in unplucked skin from the same animal (vide infra). This was true regardless of the diet or the age of the rats. No gradient of activity in skin samples progressively more removed from the site of plucking could be detected.

Effect of Age

ODC activity peaked in rats weighing 11–15 gm (aged 2–5 days) and declined irregularly thereafter (Fig 1). The differences between the 11–15 gm and 16–20 gm groups and between the 16–20 gm and 21–25 gm groups are statistically significant, with $p < 0.001$ and $0.01 < p < 0.02$, respectively. The differences between the remaining groups are not significant. With increasing age there was a steady decline in ODC activity in both types of skin (Fig 2). The increase in activity due to plucking occurred over a wide weight range and a wide range of initial (unplucked) ODC activity. In neonatal rats and rats fed a standard diet weight increased concomitantly with age.

Localization

In 3-day-old rats we found that dermis contained 10 times as much ODC activity as did epidermis (Table I). In adults the ODC levels in the dermis are lower than in neonatal dermis whether activities are expressed per gram of tissue or per milligram protein. Both epidermal and dermal ODC increased with plucking (Table I).

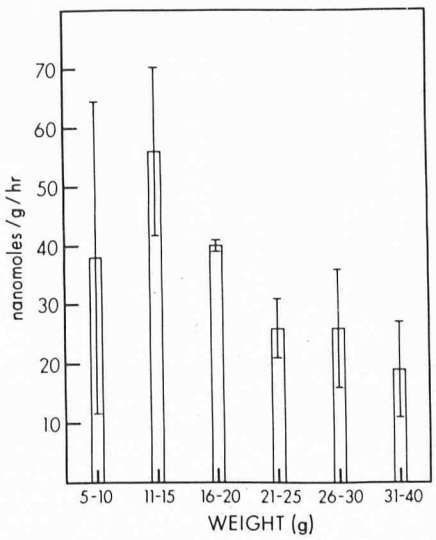

FIG 1. Skin ornithine decarboxylase activity of pre-weanling rats of different weights. The height of the *bar* represents the mean and the *vertical line* extends 1 SD above and below the mean. The skin was prepared and assayed as in Methods. There were 6, 10, 4, 2, 3, and 2 rats in the 5–10, 11–15, 16–20, 21–25, 26–30 and 31–40 gm groups, respectively.

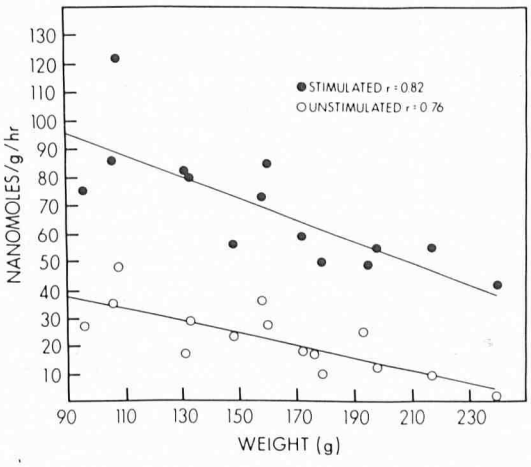

FIG 2. Skin ornithine decarboxylase activity of adult rats of different weights. All animals were fed Purina Chow. Skin was stimulated by plucking and assayed as described in Methods. Each point is the mean of duplicate assays of each piece of skin. There are both plucked and unplucked data for each animal, and the correlation coefficient (r) of weight and enzyme activity is indicated.

Heat Stability

The loss of ODC activity with preincubation at 56° followed very similar time courses for both plucked and unplucked skin. The percent activity remaining after 5, 10, 15, 20 and 30 min preincubation was, for plucked skin: 48, 43, 34, 26, and 21%, respectively; and for unplucked skin: 31, 23, 23, 12 and 9% respectively.

Mixing Experiment with Whole Skin Homogenates

The mixing experiment results were as follows: activities in 1 ml unplucked homogenate, 1 ml plucked homogenate and a mixture of ½ ml of each were: 23.6, 60.3 and 43.5 nanomoles CO_2/gm/hr, respectively. The predicted activity for the mixture was the average of the activities of the 2 pure homogenates, or 42 nanomoles/gm/hr. Thus actual and predicted activities in the mixture were within 4% of each other.

Diet Experiments

Figure 3 is a plot of average ODC activities ±1 SD in plucked and unplucked skin of rats fed for 4 days on diets containing between 2 and 24% protein (as casein). There were 3 rats in each of the 24% and 16% groups, there were 7 in the 2% group, 10 each in the 4% and 12% groups and 11 in the 8% group. ODC activities of both plucked and unplucked skin fell as the percentage of dietary protein decreased; the decrease was more gradual in plucked skin. Plucking increased ODC several fold under every regimen.

Results of studies with rats fed for 8 days on 8, 12 or 16% casein are shown in Table II. Rats on 16% casein gained more weight than rats on 12%; those on 8% gained no weight. The means of unplucked ODC activity in the 16% and 12% groups were statistically significantly different with a p value between 0.05 and 0.1. The difference between the unplucked 12% and 8% means was statistically significant with p between 0.01 and 0.025. The difference between the plucked 8% and 12% means was statistically significant ($0.25 < p < 0.05$); the plucked 12% and 16% means were not statistically significantly different ($0.20 < p < 0.25$).

No consistent differences in appearance of skin from rats maintained for 4 days on the various diets was observed by histological examination.

The recovery of ODC activity during rehabilitation after a period of protein deprivation is depicted in Fig 4. Five rats were fed on 2% protein for 7 days, then were rehabilitated with 16% casein for 2, 4, 5 or 8 days. Their ODC activities are compared to those of a rat fed on 2% casein for 4 days and to those of a rat fed 16% casein for 7 days. ODC activities in both plucked and unplucked skin were elevated after 2 days of rehabilitation. Activities seemed to plateau after 5 days refeeding, with plucked levels higher and unplucked levels the same as controls; there was a decrease with 8 days refeeding. After 8 days of refeeding plucked ODC activities were near control values and unplucked activities were lower than the control. The weight gains were (gm/day) 11, 8.8, and 10.8 for the rats refed for 2, 4 and 5 days respectively; and 8 and 10 gm/day for the rats refed for 8 days. The decreases in the rats refed for 8 days are therefore not due to differences in weight gain.

TABLE I. *Distribution of skin ornithine decarboxylase in adult and neonatal rat skin*

	Body Weight (*gm*)	Ornithine Decarboxylase Activity			
		Unplucked		Plucked	
		Dermis	Epidermis	Dermis	Epidermis
Neonate (N = 6)	—	50	5	—	—
Adult (N = 3)	119	4	4	7	37
Adult (N = 3)	180	0.9 (0.8)	1.9 (1.4)	2.7 (2.3)	19 (11.1)
Adult (N = 1)	225	2.6	0.2	29	27
Adult (N = 2)	255	0.1 (0.1)	2.0 (1.2)	7 (5.8)	30 (14.3)

Skin was plucked and heat separated and enzyme assayed as described in Methods. Activity is expressed as nanomoles/gm wet weight/hr; numbers in ( ) are the activities expressed in term of nanomoles/mg protein/hr. The skin portions are the pools of epidermis and dermis from the indicated number (N) of animals and the mean body weight of the animals is presented. The animals were fed Purina Chow.

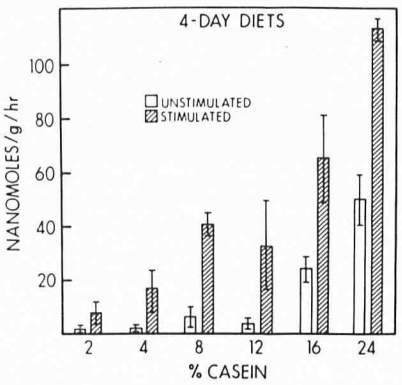

FIG 3. Relationship between the percentage of protein in the diet and skin ornithine decarboxylase activity. Animals were fed diets with 2–24% protein as described in Methods, and ornithine decarboxylase was assayed in plucked and unplucked skin. Between 3 and 11 animals were in each group (see text). The height of the *bars* represents the mean ODC activity, and the *line* in the *bars* extends 1 SD above and below the mean.

TABLE II. *Relationship of ODC activity and weight gains—8 day diets*

% Protein	Mean % Weight Gain	Ornithine Decarboxylase Activity: Mean Unstimulated	P
16	38.5	23.3	0.05–0.1
12	17.1	6.4	<0.005
8	0.1	0.3	0.01–0.025
		Mean Stimulated	
16		59.4	0.2–0.25
12		48.2	0.005–0.01
8		32.0	0.025–0.05

Mean ODC activities in unstimulated and stimulated skin from each diet group are compared to the corresponding activities in the other diet groups. Lines connect mean activities to the p values for each comparison. ODC activity is given in nanomoles/gm/hr.

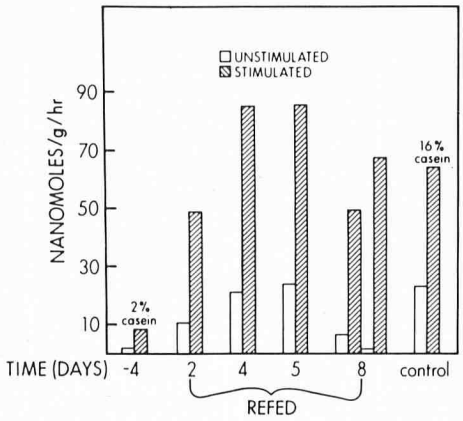

FIG 4. Relationship between the duration of refeeding and skin ornithine decarboxylase activity. Each set of *bars* except the control represents a single animal. The control is the mean value for 6 animals. Animals were on a 2% casein diet for 4 days (*−4* on the graph) and then refed a 16% casein diet 2, 4, 5 or 8 days.

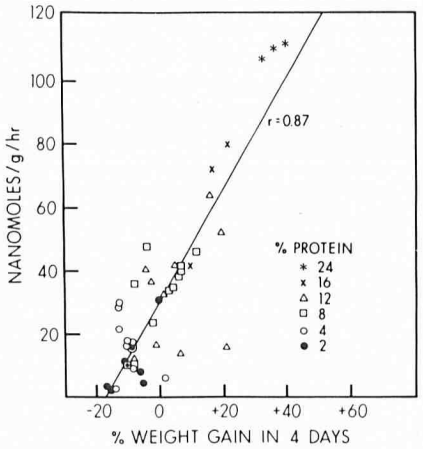

FIG 5. Relationship between weight gain and skin ornithine decarboxylase activity. Animals were fed diets with different percentages of protein for 4 days. (Same animals as in Fig 4). Weight change was highly correlated (r = 0.87) with skin ornithine decarboxylase activity.

Figure 5 is a plot of ODC activities in plucked skin vs the percent weight change of each rat during the 4-day diets. Each symbol represents a single rat on one of the special diets. There is a strong correlation between ODC activities and weight changes, with r = 0.87. In general, the higher the percentage of dietary protein the more weight the rats gained.

Kinetics

Figure 6 is a plot of ODC activity in plucked skin, expressed as disintegrations per minute of $^{14}CO_2$ vs L-ornithine concentration. The enzyme exhibited Michaelis-Menten kinetics. The *insert* is a reciprocal plot of these data. We calculated an apparent Km of 100 μM for L-ornithine. Similar experiments yielded an apparent Km of 125 uM with unplucked skin as the enzyme source.

Stoichiometry

The percentage of ornithine retention on CM-cellulose paper was 0.12%. Putrescine retention under the same experimental conditions was 35%. The amount of radioactivity retained on CM-cellulose paper spotted with the reaction mixture containing L-[^{14}C(U)]-ornithine was 6.38 pCi; the amount of ornithine in the original reaction mixture was 0.4 μCi. Thus, 0.12% of 0.4 μCi, or 0.48 pCi, of ornithine stuck to the paper. The radioactivity from putrescine retained was then 5.9 pCi. With a 35% efficiency of putrescine retention, the total radioactivity from putrescine produced was 16.85 pCi. In the parallel tube 3.99 pCi of CO_2 were trapped. The ratio of CO_2 radioactivity to putrescine radioactivity was 0.237. As uniformly labeled putrescine has 4 times as many radioactive carbons as does CO_2 produced from it, the CO_2/putrescine ratio should be 0.25 if $^{14}CO_2$ is produced only by ODC. The actual ratio is 95% of the predicted ratio (0.25). Radioactivity was found only in the polyamine fractions of the high voltage electrophoresis paper.

DISCUSSION

We have shown that skin ornithine decarboxylase is an enzyme whose level can be altered by plucking of hair and by dietary protein restriction. The results indicate that the plucking stimulus works by a method other than the removal of a soluble ODC inhibitor. No gradient of ODC activity from plucked to unplucked skin was found. Therefore, we conclude that only plucked follicles and immediately neighboring epidermis respond to this stimulus. The heat stability patterns and apparent Kms of plucked and unplucked ODC are similar; we have no reason to believe that plucking stimulates the synthesis of an isozyme of ODC different from that of unplucked skin.

The localization experiments with adult rats (Table I) and the relationship of ODC activity and age (Fig 1 and 2) indicate that skin ODC activities decrease with age. Whether this is due

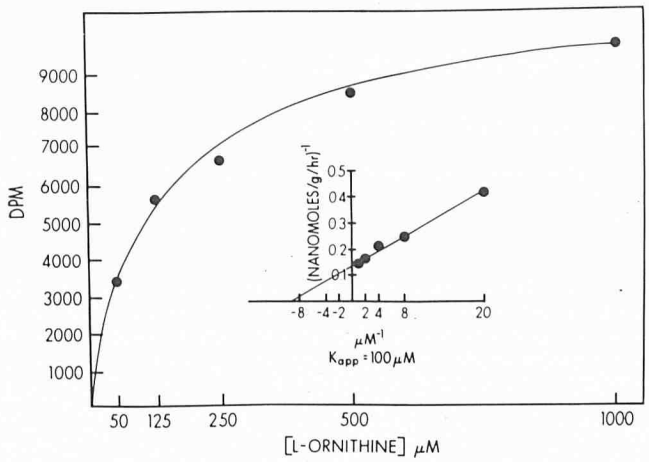

FIG 6. Enzyme kinetics of skin ornithine decarboxylase from plucked skin. The enzyme was assayed in the standard assay with varying concentrations of ornithine, all of which had constant specific activity. The *insert* is a Lineweaver-Burk plot of the data.

to decreased ODC activities in individual follicles or to fewer follicles per unit volume due to growth of the skin is not known. Probst and Krebs [18] reported that ODC activities of mouse skin follicles change cyclically, in parallel with cyclic hair growth, but do not describe an overall decrease in activity with age. The ODC activity in neonatal dermis is higher than in the epidermis; that dermal activities eventually decrease until epidermal activities are higher is suggested by the data in Table I. Thus epidermal ODC activities are more constant throughout life, which is probably a reflection of the rather constant pattern of epidermal proliferation and the number of cells in the germinative population.

Both dermal and epidermal ODC are stimulated several fold by plucking. Chase [19] reported that plucking of club hairs causes a burst of mitotic activity in "all of the epithelial components," which includes the basal layer of the epidermis. Hamilton and Potten [20] reported that plucking by the plastic dressing technique removes some 13–21% of mouse epidermal cells. After 24 hr the labeling index of epidermal sheets increased 3-fold and the turnover time for epidermal basal cells decreased by 75%. Our finding that plucking stimulates both epidermal and dermal ODC is consistent with these data.

The results of our diet experiments provide good evidence that skin ODC levels correlate well with the amount of protein in the diet and with the amount of weight lost or gained by rats. In rats, measurements of unplucked activities could differentiate degrees of protein deficiency down to about 8% protein in the diet. When dietary protein was below 8%; unplucked activities were close to zero and plucked activities would be a more accurate measure of protein malnutrition. If these studies can be extrapolated to man, the ODC assay could be useful in the diagnosis of human protein malnutrition because intermediate degrees of malnutrition could be distinguished. Plucking hair from resting follicles would increase follicular ODC, which could be assayed from a biopsy within a few hours.

The rapid response of ODC to dietary rehabilitation (Fig 4) could be used as a further test for malnutrition. Remission of other symptoms of protein deficiency (e.g. depigmentation of hair, muscular atrophy) would require more than 2 days of rehabilitation, and would not respond at all to increases in dietary protein if caused by a different pathological condition.

McAnulty and Williams [21] have studied the responses of ODC in liver, spleen, gastrocnemius and quadriceps muscle of male and female rats during dietary rehabilitation (following a period of food restriction to maintain no weight change). When free access to food was restored, ODC in liver peaked in a few hours, but peaked only after 4 or 5 days in the other organs. In no tissue did the recovery peak in ODC activity represent more than a 4-fold increase over the activities during food restriction. In contrast, skin ODC activities increased 8-fold by day 5 of rehabilitation (Fig 4).

There is a strong correlation between stimulated ODC activities and the amount of growth undergone by rats (Fig 5), and a variety of evidence suggests that ODC activity and polyamine synthesis are required for growth and cell division. The rapidity of the increase in ODC has made it applicable to other conditions in which the epidermis is stimulated, e.g. by phorbol esters in cocarcinogen experiments [2–4] and no doubt it will be useful in studying the response of the epidermis to agents such as drugs and ultraviolet light.

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